Susceptibility of Rats to Mammary Gland Carcinogenesis by the Food-Derived Carcinogen 2-Amino-1-Methyl-6-Phenylimidazo[4,5-*b*]Pyridine (PhIP) Varies with Age and Is Associated with the Induction of Differential Gene Expression

Liang Shan,* Minshu Yu,* Herman A.J. Schut,[†] and Elizabeth G. Snyderwine*

From the Chemical Carcinogenesis Section,* Laboratory of Experimental Carcinogenesis, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland; and the Department of Pathology,[†] Medical College of Ohio, Toledo, Ohio

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a heterocyclic amine found in cooked meat, induces mammary gland cancer when administered to adolescent female rats (43-day-old). In contrast, mature virgin rats (150-day-old) were resistant to mammary carcinogenesis by PhIP. To explore the possible mechanisms for the age-related differences in susceptibility, PhIP-DNA adduct levels, mutations, and gene expression were examined in glands from 43-day and 150-day-old PhIP-treated rats. In rats of different ages, PhIP-DNA adduct levels detected by the ³²P-post-labeling assay and mutant frequency measured in the *lacI* reporter gene of Big Blue rats were not statistically different. PhIP-DNA adduct levels, adduct removal, and mutation burden did not appear to account for the variation in carcinogen susceptibility with age. However, cDNA microarray analysis indicated that PhIP treatment differentially altered the profile of gene expression in glands from 43-day-old and 150-day-old rats. In 150-day-old rats, PhIP enhanced the expression of genes associated with differentiation (eg, β -casein, κ -casein, whey acidic protein) and induced morphological differentiation. In contrast, in 43-day-old rats, PhIP inhibited the expression of differentiation genes and enhanced cellular proliferation. From 3 hours to 6 weeks after PhIP dosing, the number of clones showing altered expression declined more than 50% in 150-day-old rats but increased fourfold in 43-day-old rats (29 clones *versus* 194, respectively) suggesting that PhIP induced a cascade of gene expression alterations only in susceptible rats. Genes showing altered expression specifically in 43-day-old rats included the Ras superfamily genes and genes associated with protein synthesis/degradation (lysosomal proteins, heat shock pro-

teins, and proteasomes). The microarray data support the notion that the mechanism of age-dependent susceptibility to mammary gland cancer is largely associated with differential responses in expression of genes involved in cellular differentiation, proliferation, and protein homeostasis. (Am J Pathol 2004; 165:191–202)

The mammary gland is a dynamic organ during a woman's lifetime that undergoes growth, differentiation, and regression in association with endocrinological and reproductive factors. Breast cancer is generally considered to arise as a consequence of disruption in the normal cellular pathways involved in gland development and differentiation.^{1,2} Although breast cancer is largely a disease presenting in older women, the initiation of carcinogenesis may occur in younger years when the mammary gland is most susceptible.^{3–5} Studies of atomic bomb survivors and patients receiving therapeutic ionizing radiation have shown that ionizing radiation is more apt to increase breast cancer risk when exposure occurs during childhood, adolescence, and young adulthood rather than after the age of forty.^{6,7}

The rat is a longstanding model for studying the influence of age and mammary gland development on breast cancer susceptibility and for providing insight into human breast carcinogenesis.^{5,8} The impact of age on mammary gland cancer susceptibility in rats was first recognized by Huggins.⁸ Using the chemical carcinogen 3-methylcholanthrene, mammary cancer incidence was shown to be highest in rats treated during adolescence (roughly 35 to 55 days of age) and to decline sharply after 100 days of age. The age-related decline in susceptibility of rats to mammary gland cancer has also been observed with other carcinogens including 7, 12-dimethylbenz[*a*]anthracene (DMBA) and N-methylnitrosourea

Accepted for publication March 26, 2004.

Address reprint requests to Elizabeth G. Snyderwine, Chemical Carcinogenesis Section, Laboratory of Experimental Carcinogenesis, Building 37, Room 4146, National Cancer Institute, NIH, Bethesda, MD 20892-4262. E-mail: elizabeth_snyderwine@nih.gov.

(NMU).^{9–12} Several studies have reported complete resistance to DMBA and NMU-induced mammary cancer in 150-day-old rats.^{10,12}

The mechanisms of age-dependent susceptibility to mammary cancer in rats has been under investigation for many years.^{13–24} Studies with DMBA have suggested that highly susceptible adolescent rats show higher metabolic activation, greater DNA adduct formation, and poorer mammary epithelial cell DNA repair than older, less susceptible rats.^{14–19} The mammary gland from adolescent rats was also reported to have a high labeling index that would ultimately contribute to greater cancer risk^{9,10,20}. The rate of cell proliferation in the mammary gland was shown to decline with age and be lowest at about 150 days of age.¹⁰ Russo and colleagues further demonstrated that susceptibility to carcinogenesis is associated with the developmental stage of the mammary gland at the time of carcinogen administration.^{9,21–23} Specifically, susceptibility was correlated with the percentage of terminal end buds (TEBs) in the mammary gland. Adolescent rats have relatively high levels of TEBs whereas very few TEBs can be found in mature virgin rats (140 to 180 days of age).²² With maturation, TEBs differentiate to alveolar buds that ultimately give rise to lobules. TEBs were considered to be highly susceptible to carcinogenesis because, in comparison to more differentiated structures, TEBs have the highest rate of cellular proliferation.^{9,22,23}

Although considerable progress has been made in identifying and understanding age-related susceptibility to chemical carcinogenesis in rats, the molecular basis for this phenomenon remains largely undefined. Studies to date point primarily to differences in DNA adduct formation, DNA repair, and cellular proliferation, as an explanation for the age-related differences in susceptibility. Interestingly, it has been recently proposed that resistance to mammary gland carcinogenesis in parous rats may be associated with an altered expression of genes regulating the intracellular pathways governing proliferation responses to carcinogens.^{24,25} No study has yet addressed whether the mammary gland from adolescent and mature rats shows different gene expression responses after chemical carcinogen exposure. Furthermore, defining these carcinogen-induced changes in gene expression may ultimately provide a molecular profile for susceptibility and resistance to mammary gland carcinogenesis.

In the current study, we used cDNA microarray analysis to examine the expression of genes in the mammary gland of adolescent and mature rats after chemical carcinogen exposure. We have hypothesized that the molecular/cellular responses to a mammary gland carcinogen are different between the susceptible and resistant rats of different ages, and accordingly, that distinct gene expression patterns would be induced by a chemical carcinogen. Herein we used 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) a chemical carcinogen found in the human diet in cooked meat that is a well-recognized mammary gland carcinogen in rats and a suspected human carcinogen.^{26,27} These studies were also undertaken to provide insight into the earliest mo-

lecular alterations associated with neoplastic transformation and resistance in the mammary gland.

Materials and Methods

Animals, PhIP Treatment, and Mammary Gland Carcinogenesis

Female Sprague-Dawley rats were obtained from the National Institutes of Health (NIH) animal supply (Animal Production Area, Frederick, MD). For mutagenesis studies, Blue transgenic rats carrying the *lacI* mutational reporter gene were purchased from Taconic Laboratories (Germantown, NY). All animals were provided NIH Lab Chow and water *ad libitum* and housed in a NIH animal facility on a 12-hour light/12-hour dark cycle. Adolescent (43-day-old) and mature (150-day-old) rats were administered 10 oral doses of PhIP-HCl (75 mg/kg) (Toronto Research Chemicals, North York, ON) once per day over a 12-day period as described previously.^{28,29} The control rats were administered vehicle only (water). Rats were euthanized 3 hours after the final dose of PhIP or placed on defined high-fat diet²⁸ for 6 weeks before euthanasia. Big Blue rats were autopsied at the 6-week time point only. On autopsy, the mammary gland samples were taken and snap-frozen in liquid nitrogen and stored at -80°C before isolation of DNA or RNA. Previous studies indicated no differences in mutant frequency between whole mammary gland and isolated mammary epithelial cells. For histological examination, a portion of the fourth gland was fixed in 10% formalin. Mammary glands (numbers 4 to 6) were used for nucleic acid isolation after lymph nodes were removed by dissection. For carcinogenesis studies, 43-day-old and 150-day-old female Sprague Dawley rats (20 each) were administered the same 10-dose regimen of PhIP and placed on defined high-fat diet for 6 months.^{28,29} Rats were palpated weekly and autopsied as described previously.²⁹

RNA and DNA Isolation

High molecular weight DNA was isolated by the phenol/chloroform method. Total RNA was isolated using TRIzol extraction reagent (Invitrogen, Carlsbad, CA) with double precipitation according to the manufacturer's protocol.

Mutagenesis Assay

The *lacI* mutant frequency in mammary gland from PhIP-treated Big Blue rats was determined using the methods outlined by Stratagene (La Jolla, CA) as described in detail previously.³⁰

DNA Adduct Analysis

PhIP-DNA adduct levels were determined by ³²P-post-labeling assay using intensification (ATP-deficient) conditions.^{31,32} The method resolves PhIP-DNA adducts as [³²P]ATP-labeled bis-phosphonucleotide adduct finger-

prints on autoradiograms after chromatography. Adduct levels were quantified by Cerenkov counting, and DNA adduct levels expressed as relative adduct labeling (RAL).

cDNA Microarrays, Probes, and Hybridizations

Microarray analysis was carried out on a mouse cDNA microarray containing 9984 cDNA clones (National Cancer Institute, Bethesda, MD).^{33,34} The use of the mouse cDNA microarray for rat has been previously validated in our laboratory (Ju-Seog Lee, personal communications). Thirty micrograms of total RNA were used to synthesize cDNA by oligo(dT)-primed polymerization using SuperScript II reverse transcriptase and labeled by Cy3 (Cy3) and Cy5 (Cy5) mono-reactive dyes (Amersham Biosciences, Buckinghamshire, England). The labeled Cy3 and Cy5 cDNA probes were purified and concentrated on a Microcon column YM-30 (Millipore, Bedford, MA), achieving a final volume of 15 μ l. Probes were mixed with 1 μ l of Cot-1 DNA, 1 μ l of yeast t-RNA and 1 μ l of poly(A), denatured for 1 minute at 100°C and then mixed with 18 μ l of 2X hybridization buffer (50% formamide, 10X SSC, 0.2% SDS). Slides were first pre-hybridized with 30 μ l pre-hybridization buffer (5X SSC, 0.1% SDS, and 1% bovine serum albumin in TE buffer) for 1 hour, rinsed with H₂O for 2 minutes, and dehydrated in isopropyl alcohol for 2 minutes. Hybridization was carried out by adding the probe to the array and incubating overnight at 42°C. After washing, the slides were scanned using the Axon GenePix 4000 scanner and GenePix Pro 3.0 software (Axon, Union City, CA). For data normalization, interpretation, and visualization, the image and raw data were deposited into the NCI microarray database system supported by the Center for Information Technology (<http://nciarray.nci.nih.gov>). For arrays comparing gene expression in 43-day and 150-day untreated rats, mammary gland RNA from five rats in each age group were pooled and hybridization was carried out three times. For arrays comparing gene expression in PhIP-treated and control rats in each age group, control rat mammary gland RNA within each age group ($N = 5$ control rats) was pooled before hybridization against individual age-matched PhIP-treated rat ($N = 3$) mammary RNA samples.

Microarray Data Normalization and Analysis

Total intensity normalization [50th percentile (median)] was used to correct bias caused by systematic differences. Mean target intensity minus median background was used for signal calculation. Each clone intensity was re-scaled by a normalization factor such that the median fluorescence ratio of all genes was 1.0. Changes in gene expression were presented as logarithmic ratios of fluorescence intensities [channel B/channel A (Cy5/Cy3)]. The spots were filtered for both Cy3 and Cy5 channels by such that the minimum percentage of target pixels was at least one standard deviation above the background, the signal intensity above background was at least two standard deviations, the signal intensity to background ratio

was at least one, and the minimum absolute signal intensity was at least 500. The criteria for selecting a differentially expressed clone was that it showed relative to control greater than a twofold expression change in at least two of the three replicates and greater than a 1.5-fold expression change in the third replicate. The data were further analyzed by hierarchical clustering (Stanford's Cluster and TreeView Program and BRB array tools) available from the NCI microarray database website (<http://nciarray.nci.nih.gov>).

Semi-Quantitative RT-PCR

One microgram of total RNA was used to synthesize the first-strand cDNA in a final volume of 20 μ l using SuperScript First-Strand Synthesis Kit (Invitrogen). Then, 0.5 μ l of the cDNA was used to amplify a segment of the α -casein (*Csn1*), β -casein (*Csn2*), κ -casein (*Csn10*), whey acidic protein (*Wap*), *RAN*, *RAB11a*, and Ras-GTPase-activating protein SH3-domain binding protein (*G3bp*) separately using the following specific primers: *Csn1*-F, tcgtcaacaacctgaa-cagac and *Csn1*-R, tccactacactcataggtgtc; *Csn2*-F, ag-gatgcattcactgtgtc and *Csn2*-R, aggtcttgaaacaggcatac; *Csn10*-F, agaactgactccgtgtgaag and *Csn10*-R, accact-gactctgtggtag; *Wap*-F, tgcctcatcagcctcgtc and *Wap*-R, tct-ggatccaagagtcaag; *G3bp*-F, tggtaaccaggagatgtg and *G3bp*-R, tctgagccaagcaattcac; *Ran*-F, tcgtctccataccaa-cag and *Ran*-R, acaggctgcctatcctcatc; *Rab11a*-F, agattctg-gtgtgggaag; and *Rab11a*-R, accacattgttgcttgag. *GAPDH* was amplified as the internal control using primers purchased from Invitrogen. PCR was carried out for 20 to 25 cycles with each cycle including denaturing for 45 seconds at 94°C, annealing for 45 seconds at 58°C, and polymerization for 45 seconds at 72°C. The PCR product was separated on 2% agarose gel containing ethidium bromide (EtBr) and photographed under UV light.

Statistical Analysis

The statistical analysis in Figure 1 was carried out with SigmaStat 2.0 statistical software (Jandel Scientific Software, San Rafael, CA).

Results

DNA Adducts, Mutagenicity, and Carcinogenicity of PhIP in 43-Day-Old and 150-Day-Old Rats

PhIP-DNA adduct levels in mammary gland from 43-day-old and 150-day-old Sprague-Dawley rats treated with PhIP were measured by the ³²P-post-labeling assay (Figure 1A). At either 3 hours and 6 weeks after PhIP dosing, adduct levels were not statistically different ($P > 0.05$), although there was a trend toward higher adduct levels in older rats. In rats of both ages, PhIP-DNA adduct levels declined to approximately 10 to 20% the 3-hour value by 6 weeks. The profile of adducts was identical in rats of both ages and identical to that reported previously.³²

Figure 1. PhIP-DNA adduct levels (A) and mutant frequency (B) in rats of different ages. DNA adduct levels were determined by the ^{32}P -post-labeling method and expressed as relative adduct labeling (RAL). Values are the mean $\pm$ SE of three to four rats. Differences between bars were not statistically significant (Student's *t*-test, $P > 0.05$, two-tailed). *P* values are listed above bars.

Mutagenicity in the mammary gland was also assessed in 43-day-old and 150-day-old Big Blue rats 6 weeks after PhIP treatment (Figure 1B). At both ages, mutant frequency was 7- to 11-fold higher in PhIP treated rats than in corresponding age-matched controls. However, there were no statistical differences in mutant frequency between 43-day-old and 150-day-old rats given PhIP ($P > 0.05$). The mutant frequency in 43- and 150-day-old control rats was also not statistically different. The mammary gland carcinogenicity of PhIP was further compared in 43- and 150-day-old Sprague-Dawley rats given identical dosage regimens. Whereas nearly 50% of 43-day-old rats develop mammary gland tumors within 6 months of PhIP treatment,²⁹ no mammary gland tumors were detected within this same time period in 150-day-old rats.

Mammary Gland Gene Expression in 43-Day-Old and 150-Day-Old Rats

The known morphological and developmental differences in mammary glands from adolescent and mature rats suggested that gene expression profiles would also be different. To test this hypothesis, gene expression profiles were examined by cDNA microarray analysis in mammary gland from 43- and 150-day-old rats. A total of 69 clones were differentially expressed (≥ 2.5 fold) in-

Figure 2. Differential gene expression in 43-day-old and 150-day-old normal rats. **A-E:** Unique clusters of genes with related functions and/or similar changes in expression. **A** represents genes with higher expression in 43-day-old rats. **B-E:** Genes with higher expression in 150-day-old rats that are functionally associated with differentiation, cell growth/apoptosis/repair, metabolism and cytoskeleton/adhesion/extracellular matrix, respectively. The assay was carried out using a cut-off value of ≥ 2.5 -fold change in at least two of the three analyses and ≥ 1.5 -fold change in the third hybridization. Forty three-day-old rats were labeled by cyanine 3 and 150-day-old rats by cyanine 5. **Green squares**, clones with high relative expression in 43-day-old rats; **black squares**, clones with similar expression (ratio of approximately 1); **red squares**, clones with high relative expression in 150-day-old rats; **gray squares**, insufficient data.

cluding 62 known genes and seven unknown clones. The known genes were further grouped into several categories depending on function and relative expression level (Figure 2, A to E). Only eight genes showed relatively low expression in 150-day-old rats (ie, showed relatively high expression in 43-day-old rats) (Figure 2A). The majority of differentially expressed genes ($N = 61$) showed relatively high expression in 150-day-old rats suggesting that mammary gland development was associated with a general increase in gene expression, at least with the array used here. Genes showing differential expression were associated with differentiation (Figure 2B), cell growth/apoptosis/repair (Figure 2C), metabolism (Figure 2D), and cytoskeleton/adhesion/extracellular matrix (Figure 2E). The higher expression of genes related to mammary gland differentiation such as α -, β -, κ -casein, milk fat globule-EGF factor 8 protein, and the N-myc downstream regulated 1 gene in 150-day-old rats is consistent with the more advanced state of differentiation in the mature rat.³⁵⁻³⁸

Table 1. Known Genes Showing Altered Expression in Mammary Glands of 43-Day-Old Rats 3 Hours after PhIP Treatment

Underexpressed	Overexpressed
Differentiation apolipoprotein C-II casein beta casein kappa whey acidic protein butyrophilin, member A1 LIM domain only 4	Cell growth/apoptosis/repair RAS-related C3 botulinum substrate 2 ras homolog gene family, member E Rho guanine nucleotide exchange factor 3 mutY homolog transgelin peptidylprolyl isomerase C secretory leukocyte protease inhibitor glypican 3
Cell growth/apoptosis/repair metallothionein 2 ras homolog gene family, member A v-rat simian leukemia viral oncogene homolog B ribosomal protein L36 insulin-like growth factor binding protein 2 nuclear factor, erythroid derived 2, like 3	Cytoskeleton/adhesion/extracellular matrix lumican bystin-like decorin
Cytoskeleton/adhesion/extracellular matrix capping protein, gelsolin-like	Metabolism stearyl-Coenzyme A desaturase 1 Golgi autoantigen, subfamily a, 4
Metabolism Tcfcp2-related transcriptional repressor 1 solute carrier family 5, member 6 serine/threonine kinase 2	Miscellaneous immunoglobulin kappa chain variable 8 immunoglobulin joining chain immunoglobulin heavy chain

Thirteen under-expressed and 2 over-expressed unknown clones are not included in the table.

Mammary Gland Gene Expression in 43-Day-Old and 150-Day-Old Rats Exposed to PhIP

Expression 3-Hours after PhIP Treatment

In 43-day-old rats, 47 clones including 32 known genes and 15 unknown sequences showed altered expression (≥ 2.0 fold) 3 hours after the carcinogenic dose regimen of PhIP was administered. Twenty-nine clones (16 known genes and 13 unknown clones) were under-expressed and 18 (15 known and 3 unknown) were over-expressed in mammary gland from PhIP-treated rats *versus* aged-matched control. Known genes showing altered expression were categorized as associated with differentiation, cell growth/apoptosis/repair, cytoskeleton/adhesion/extracellular matrix, and metabolism (Table 1). Multiple milk protein genes such as β -casein, κ -casein, whey acidic protein, and butyrophilin (subfamily 1, member A1) showed reduced expression after PhIP treatment suggesting an inhibition of differentiation.^{35,39,40} Glands from 43-day-old rats treated with PhIP also showed changes, both increases or decreases, in the expression of genes related to cell growth/apoptosis/repair. In this category, five genes were functionally related to the Ras superfamily.⁴¹ Three of the five genes including the ras homolog gene family member (*Rho*) E, Ras-related C3 botulinum substrate (*Rac*) 2, and Rho guanine nucleotide exchange factor (*GEF*)3 showed a 2- to 3-fold higher expression, whereas the *ras* homolog gene family member (*Rho*) A and *v-rat* simian leukemia viral oncogene homolog showed at least a 50% reduction in expression in PhIP-treated rats than in controls.

Gene expression in the mammary gland was also examined in 150-day-old rats treated with the same dose regimen of PhIP. Microarray analysis compared the ex-

pression in glands from carcinogen treated and control 150-day-old rats. A total of 70 clones were abnormally expressed (≥ 2 fold), including 61 known genes and nine unknown sequences. The overwhelming majority of these clones ($N = 64$) were overexpressed, and relatively few ($N = 6$) were underexpressed after PhIP treatment (Table 2). The known genes that were overexpressed comprised four major categories. The largest group of genes was metabolism-related followed by a group of genes associated with cell growth/apoptosis/repair. Nine known genes associated with cytoskeleton/adhesion/extracellular matrix were found to be overexpressed. Notably, multiple genes associated with differentiation were overexpressed in 150-day-old rats after PhIP exposure. These data indicated that PhIP treatment augmented mammary gland differentiation in 150-day-old rats.

Cluster analysis revealed that the alterations in gene expression induced by PhIP in mammary glands from 43-day-old and 150-day-old rats were distinct (Figure 3A). Genes associated with differentiation clearly were differentially expressed between 43-day-old and 150-day-old rats by microarray analysis (Figure 3B). As confirmed by RT-PCR analysis, PhIP exposure reduced the expression of genes associated with differentiation in 43-day-old rats but increased the expression of many of these same genes in 150-day-old rats (Figure 3, C and D).

Expression 6 Weeks after PhIP Treatment

The 6-week time point was chosen for microarray analysis to assess changes in gene expression in histologically normal mammary gland just before cancer development. Mammary tumors are rarely seen at this time point, and none of the rats in this study showed palpable tumors. Preneoplastic alterations are not readily observed 6

Table 2. Known Genes Showing Altered Expression in Mammary Glands of 150-Day-Old Rats 3 Hours after PhIP Treatment

Underexpressed	
selenoprotein P, plasma, 1	hemoglobin, beta, adult major chain
Ia-associated invariant chain	stearoyl-Coenzyme A desaturase 1
albumin 1	histocompatibility 2, class II antigen E alpha
Overexpressed	
Differentiation	
casein kappa	casein alpha
casein beta	milk fat globule-EGF factor 8 protein
midkine	butyrophilin, subfamily 1, member A1
whey acidic protein	SRY-box containing gene 9
Cell growth/apoptosis/repair	
disabled homolog 2	basic leucine zipper and W2 domains 2
G0/G1 switch gene 2	platelet derived growth factor, alpha
inhibitor of DNA binding 2	insulin-like growth factor binding protein 2
ets homologous factor	lysosomal-associated protein transmembrane 4B
ribosomal protein S19	high mobility group nucleosomal binding domain 2
metallothionein (1 and 2)	X-ray repair complementing defective repair in Chinese hamster cells 1
Cytoskeleton/adhesion/extracellular matrix	
actin (Actg2, Acta2)	myosin heavy chain 11
dystroglycan 1	serine protease inhibitor, kunitz type 2
calponin 1	tropomyosin 2, beta
tubulin, beta 5	procollagen (Col2a1, Col1a2, Col1a1, Col12a1)
keratin complex (Krt1-19, Krt2-5)	
Metabolism	
carbonic anhydrase 2	hydroxyacyl-Coenzyme A dehydrogenase type II
retinol binding protein 7	poly A binding protein, cytoplasmic 2
acarnitine palmitoyltransferase 1	potassium channel, subfamily K, member 1
ATPase (Atp6v0d1, Atp2a1)	glutathione S-transferase, alpha 3
glutamate-ammonia ligase	GPI-anchored membrane protein 1
adenylosuccinate synthetase 1	solute carrier family 30, member 4
creatinine kinase, muscle	O-6-methylguanine-DNA methyltransferase
H3 histone, family 3B	inosine 5'-phosphate dehydrogenase 2
peroxiredoxin 2	
Miscellaneous	
CD 81 antigen	CD 24a antigen

Nine over-expressed unknown clones are not included in the table.

weeks after PhIP in 43-day-old rats.⁴² Furthermore, PhIP and PhIP metabolites have been largely cleared from the rat at this time point, and PhIP-DNA adduct levels have declined but mutations have been induced. Microarray analysis of the gland from PhIP-treated 43-day-old rats showed a surprisingly high number of overexpressed clones ($N = 194$) including 168 known genes and 26 unknown sequences (Tables 3 and 4). The majority of clones that satisfied our selection criteria showed a modest 2- to 3-fold increase in expression. The majority of overexpressed genes were associated with cell growth/apoptosis/DNA repair (Table 3). Increased expression of proliferating cell nuclear antigen (*PCNA*) and lipocalin 2, a putative estrogen target gene,⁴³ suggested that there was increased proliferation in the mammary gland. Other categories of overexpressed genes were associated with metabolism and cytoskeleton/adhesion/extracellular matrix. Also observed was an increased expression of genes associated with protein transport across the endoplasmic reticulum including *SEC61* and translocating chain associated membrane protein 1.^{44,45}

Many of the overexpressed genes detected in 43-day-old rats at 6 weeks fell into distinct gene groups or families (Table 4). The largest family of genes included ribosomal proteins and ribosome-associated proteins. Forty-one ribosomal protein transcripts showed higher expression 6 weeks after PhIP treatment than in controls. Other gene families showing overexpression included proteasomes/protein degradation-related genes, as well as the heat shock proteins and chaperonin subunits. At this time point, many genes in the Ras superfamily and related genes also showed an increase in expression in PhIP-treated rats relative to control (Table 4 and Figure 4A).

In contrast to the high number of clones overexpressed in 43-day-old PhIP-treated rats at 6 weeks, only 29 clones were overexpressed in 150-day-old PhIP-treated rats at this same time point (Table 5). As was observed in 43-day-old rats, however, only overexpressed clones were detected in 150-day-old rats. In 150-day-old rats at 6 weeks, PhIP treatment was associated with an overexpression of differentiation-associated genes, a finding that was consistent with the results from

Figure 3. Hierarchical clustering of cDNA microarray expression using uncentered correlation and average linkage and semi-quantitative RT-PCR analyses 3 hours after PhIP dosing. The microarray analysis was performed with RNA from PhIP-treated rats as the test samples (labeled by cyanine 5) and RNA from corresponding controls as the reference (labeled by cyanine 3). **A:** Hierarchical cluster analysis based on 835 genes (that met the selection criteria outlined in Materials and Methods) from PhIP-treated 43-day-old rats (43D-P1 to 3) and PhIP-treated 150-day-old rats (150D-P1 to 3). The figure illustrates that distinct gene expression profiles were induced by PhIP in rats of different ages. **B:** An expansion of a representative cluster (marked with a bar in **A**) containing clones associated with mammary gland differentiation such as α -, β -, κ -casein, and whey acidic protein. The expression of these differentiation associated genes was inhibited by PhIP in 43-day-old rats but induced in 150-day-old rats. **C** and **D:** Semi-quantitative RT-PCR analysis of α -, β -, κ -casein, and whey acidic protein consistent with the microarray analysis.

the 3-hour time point. The overwhelming majority of changes in gene expression induced by PhIP were distinct between 43-day-old and 150-day-old rats (compare Tables 3, 4, and 5). Multiple genes in the Ras superfamily and associated with Ras were differentially expressed between 43-day-old and 150-day-old rats (Figure 4A). The expression of several of these genes was further examined by RT-PCR in 43-day-old and 150-day-old rats (Figure 4, B and C). *Ran*, Ras-GTPase-activating protein SH3-domain binding protein (*G3bp*), and *Rab11a* showed on average a 2- to 3-fold increase in expression in PhIP-treated 43-day-old rats in comparison to 43-day-old control, but little to no change in expression was detected by RT-PCR in PhIP-treated 150-day-old rats relative to controls. Expression levels of cytokeratins 5, 8, 14, and 18 were not different between glands from control and PhIP-treated rats indicating that the observed changes in gene expression were not a consequence of a change in the size of the epithelial cell compartment.

Mammary Gland Histology after PhIP Exposure

Mammary glands from 150-day-old rats treated with PhIP had an increased number of alveoli and ducts, dilation of the ducts, and an accumulation of secretory products within the ducts and alveoli as revealed under light microscopy (Figure 5). The morphological appearance of the glands from PhIP-treated rats was similar to early pregnant mammary glands and representative of greater differentiation in comparison to control glands from 150-day-old rats. The morphological changes in glands from 150-day-old rats were more pronounced at 6 weeks than at 3 hours after PhIP. In contrast, no histological changes were evident in hematoxylin-eosin (H&E) stained sections of glands from 43-day-old rats treated with PhIP (data not shown), although alteration in the percentage of terminal end buds and alveolar buds have been previously detected in mammary gland whole mounts.⁴⁶

Discussion

The current study examined the mechanisms for the age-related susceptibility to chemically-induced rat mammary gland cancer using PhIP, a heterocyclic amine found in the human diet. Whereas 43-day-old rats were susceptible to PhIP-induced carcinogenesis, 150-day-old rats were resistant. We initially addressed whether the age-related difference in susceptibility to PhIP was associated with differences in mammary gland DNA adduct levels. Previous studies suggested that PhIP-DNA adduct levels in the mammary gland correlate with tumor incidence.⁴⁷ Nevertheless, PhIP-DNA adduct levels were not statistically different between 43- and 150-day-old rats ($P > 0.05$) and even tended to be higher in 150-day-old rats. In rats of both ages, adduct levels were similarly reduced from 3 hours to 6 weeks after PhIP. Therefore, the age differences in susceptibility to PhIP-induced mammary gland cancer did not appear to be related to differences in adduct levels or adduct removal.

Mutagenesis in the mammary gland was also assessed in Big Blue (*lacI*) transgenic rats of different ages. The induction of mutations is determined by multiple factors including initial DNA adduct levels, DNA repair, and cellular proliferation.^{48,49} Since the rate of proliferation in the mammary gland is known to be higher in 43-day-old rats than in 150-day-old rats,¹⁰ we had anticipated that mutant frequency in the *lacI* reporter gene would also be greatly elevated in 43-day-old rats. However, there was no statistical difference in mutant frequency between the age groups. The overall load of mutations in the mammary gland did not appear to explain the age-related variation in mammary gland cancer susceptibility to PhIP.

The mammary glands from 43-day-old and 150-day-old rats were, however, different with respect to gene expression profiles. Consistent with the known morphological and developmental differences observed in rats of these ages,^{5,9,50} the expression of differentiation-related genes was higher in the mammary gland from 150-day-old rats (Figure 2). In light of these molecular differences

Table 3. Known Genes Found Overexpressed in Mammary Glands of 43-Day-Old Rats 6 Weeks after PhIP Treatment

Cell growth/apoptosis/repair	
split hand/foot deleted gene 1	retinoblastoma binding protein 4
cellular nucleic acid binding protein	arginine-rich, mutated in early stage tumors
Bcl2-associated athanogene 1	CDK2-associated protein 1
DEAH box polypeptide 15	death-associated kinase 3
enhancer of rudimentary homolog	eukaryotic translation initiation factor 3 (5, 7, 8)
exportin 1, CRM1 homolog	poly (A) polymerase alpha
interferon receptor 2	splicing factor, arginine/serine-rich 2
numb gene homolog	papillary renal cell carcinoma
proliferating cell nuclear antigen	small nuclear ribonucleoprotein (D1, E, F, G)
quaking	zinc finger protein (44, 100, 148, 3612)
matrin 3	G1 to phase transition 1
guanine nucleotide binding protein, beta 2, related sequence 1	single-stranded DNA binding protein 3
poly(rC) binding protein 1	TATA box binding protein-like 1
TAF10 RNA polymerase II	RNA polymerase II transcriptional coactivator
Cbp/p300-interacting transactivator	RNA polymerase B transcription factor 3
Telomerase binding protein, p23	high mobility group nucleosomal binding domain 1
endothelin 1	high mobility group nucleosomal binding domain 2
	lipocalin 2
Cytoskeleton/adhesion/extracellular matrix	
actin, beta, cytoplasmic	amelogenin X chromosome
tubulin, alpha 2	cyclic nucleotide phosphodiesterase 1
dynein, axonemal, heavy chain 11	GPI-anchored membrane protein 1
myosin light chain, nonmuscle	synapsin I
t-complex protein 1	tropomyosin 3, gamma
Metabolism	
3-phosphoglycerate dehydrogenase	ATP synthase (Atp5b, Atp5f1, Atp5g1, Atp51)
aldolase 1, A isoform	cholinergic receptor, beta polypeptide 2
calcium binding protein, intestinal	cytochrome c oxidase (Vic, VIIa2, VIIc)
glucose regulated protein	solute carrier family 25, member 4 and 5
pyruvate kinase, muscle	stearoyl-Coenzyme A desaturase 2
thioredoxin domain containing 4	NADH dehydrogenase 1 alpha subcomplex, 2
striatin, calmodulin binding protein 4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1
Protein transport	
SEC61, gamma subunit	translocating chain-associating membrane protein 1

Twenty-six over-expressed unknown clones and 8 miscellaneous genes are not included in the table.

it appeared plausible that the molecular/cellular responses of the glands to a chemical carcinogen would also be distinct. After PhIP administration, gene expression profiles in the mammary gland were different between 43-day-old and 150-day-old rats. PhIP induced opposite changes in the expression of genes associated with cellular differentiation in younger and older rats. Specifically, α -casein, β -casein, κ -casein, and whey acidic protein were down-regulated in 43-day-old rats while up-regulated in 150-day-old rats. In accordance with the molecular changes, PhIP also clearly enhanced morphological differentiation in 150-day-old rats (Figure 5). In 43-day-old rats, the finding that PhIP down-regulated the expression of differentiation genes concurs with previous morphological studies showing that PhIP inhibited the differentiation of TEBs to alveolar buds.⁴⁶ Research by Russo and colleagues^{21-23,51} has supported that mammary cancer initiation requires the interaction of the carcinogen with undifferentiated epithelium and that differentiation inhibits cancer initiation. Our data suggest that one potential mechanism for the age-related difference in mammary cancer susceptibility involves the divergent effects of PhIP on mammary gland differentiation.

It is not yet known why PhIP stimulates differentiation in older rats but inhibits differentiation in younger rats. The duration of exposure of the gland to ovarian estrogen and progesterone, which is secreted with the onset of the estrus cycle around 35 to 42 days of age,⁵⁰ is different between younger and older rats. It is known that exposure to these hormones induces persistent changes in mammary gland gene expression and modifies responses to differentiation signals.^{25,52,53} PhIP has been shown to induce differentiation of HC11 mammary epithelial cells under specific culture conditions.⁵⁴ It appears possible that differences in the length of exposure of the adolescent and mature mammary gland to the mammatrophic hormones may in part account for the age differences in the effect of PhIP on gland differentiation.

Other changes in gene expression were also distinct between 43-day-old and 150-day-old rats after PhIP treatment. From 3 hours to 6 weeks after PhIP dosing, the number of clones showing altered expression declined more than 50% in 150-day-old rats but increased fourfold in 43-day-old rats. These data suggest that while there was a tendency for glands from older rats to recover from the PhIP exposure, there was a cascade of gene expres-

Table 4. Distinct Clusters of Genes Over-Expressed in 43-Day-Old Rats 6 Weeks after PhIP Treatment

Ras superfamily and associated genes

RAS related protein 1a
 RAS related protein 1b
 Ras-related C3 botulinum substrate2
 Rho guanine nucleotide exchange factor (GEF) 1
 v-ral simian leukemia viral oncogene homolog B
 Ras-GTPase-activating protein SH3-domain binding protein
 RAB11a
 RAB2
 RAN

Ribosomal and ribosome associated proteins

Ribosomal proteins

L3, L6, L7, L8, L10A, L12, L13, L13a, L19, L21, L22, L26, L27a, L28, L35, L35a, L36, L37a, L41, L44, S3, S3a, S4, S5, S6, S7, S2, S11, S14, S15, S17, S19, S23, S24, S29, large P2, large P1 and SA

Ribosomal associated proteins

nucleophosmin 1
 nucleolar protein family A, member 3
 acidic ribosomal phosphoprotein P0

Proteasomes and protein degradation-associated genes

Proteasomes

Psme1, Psma2, Psma6, Psmb1, Psmb2, Psmb4, Psmb6, Psmb9

Protein degradation-associated genes

ubiquitin-like 1
 ubiquitin-activating enzyme E1, Chr X
 lysosomal-associated protein transmembrane 4B

Heat shock proteins

Hspa5, Hsp105, Hspe1, Hspd1, Hspca, Hspcb, Hspa8, chaperonin (subunit 2, 4, 5, 7, 8)

Figure 4. Differential expression of genes associated with the Ras superfamily in 43-day-old and 150-day-old rats 6 weeks after PhIP treatment. **A:** Clustering of genes associated with Ras superfamily that were overexpressed in 43-day-old rats, but showed little or no change in 150-day-old rats after PhIP. **B and C:** Confirmation of microarray data by semi-quantitative analysis of selected genes *Ran*, *G3bp*, and *Rab11a*. The PhIP-treated 43-day-old and control rat samples were labeled as 43D-P1 to 3 and 43D-C, respectively. The PhIP-treated 150-day-old and control rat samples were labeled as 150D-P1 to 3 and 150D-C, respectively.

sion alterations induced by PhIP in glands from susceptible rats.

The expression of several major groups of genes was altered in glands from 43-day-old PhIP-treated rats but generally not altered in the older rats (compare Tables 4 and 5). At 6 weeks after PhIP, 41 ribosomal protein transcripts were overexpressed in 43-day-old rats whereas only one ribosomal protein (L6) was altered in older rats. Proteasomes and protein degradation-associated genes involving ubiquitin-dependent pathways also tended to be overexpressed in the younger but not older rats. Furthermore, at 6 weeks after PhIP, a large group of heat shock proteins (seven heat shock proteins and four chaperonin subunits) were overexpressed in 43-day-old rats while only two heat shock proteins were overexpressed in older rats. All three groups of genes found altered in 43-day-old PhIP-treated rats, ribosomal proteins, proteasomes and heat shock proteins, are involved in cellular protein homeostasis.⁵⁵⁻⁶⁴ Overall, these changes suggest that PhIP exposure may be associated with alterations in the balance of protein synthesis and degradation especially in rats susceptible to mammary gland carcinogenesis.

Another category of genes showing altered expression in 43-day-old PhIP-treated rats belonged to the Ras superfamily. These genes showed little to no change in expression in the older rats. The Ras superfamily has diverse cellular functions including regulating actin cy-

toskeleton, nuclear import, apoptosis, and the cell cycle.^{41,65} The finding that multiple genes in the Ras superfamily showed increased expression in the mammary gland from 43-day-old rats but not in 150-day-old rats raises the possibility of the involvement of these genes in breast cancer susceptibility and the early stages of carcinogenesis. It is also notable that an increased expression of PCNA was detected in glands from 43-day-old PhIP-treated rats. Accordingly, we previously observed by PCNA immunostaining that PhIP enhanced the proliferation in mammary TEBs from 43-day-old rats.⁴⁶ Further studies are required to determine the contribution of specific Ras superfamily genes as well as other genes in the regulation of mammary gland cell proliferation and carcinogenesis in young PhIP-treated rats.

An increase in cellular proliferation in the mammary gland has been recognized to be an early event in rat

Table 5. Known Genes Over-Expressed in Mammary Glands of 150-Day-Old Rats 6 Weeks after PhIP Treatment

Differentiation associated	
casein alpha	casein beta
casein kappa	neuronatin
transferrin	
Cell growth/apoptosis/repair	
CDC-like kinase 2	eukaryotic translation initiation factor 5A
glutathione peroxidase 4	MAP kinase-interacting serine/threonine kinase 2
sequestosome 1	guanine nucleotide binding protein, α inhibiting 2
phosphodiesterase 10A	heat shock protein (Hspd1, Hspcb)
ribosomal protein L6	latent TGF beta binding protein 3
Metabolism	
ATP synthase (Atp5p, Atp5g3)	
phosphoenolpyruvate carboxykinase 1	
translocase of outer mitochondrial membrane 40 homolog	
Cytoskeleton/adhesion/extracellular matrix	
actin, alpha 2	dynein, axonemal, heavy chain 11
myosin light chain, alkali, nonmuscle	low density lipoprotein receptor-related protein 1
tissue inhibitor of metalloproteinase 3	
Miscellaneous	
albumin 1	

Three over-expressed unknown clones are not included in the table.

mammary gland carcinogenesis.^{21,51,66,67} A proliferative burst in mammary gland from susceptible virgin rats but not parous (resistant) rats has been observed soon after NMU treatment.²⁴ Our data with younger and older rats that differ in cancer susceptibility concur with the notion that a proliferative response is an early event in carcinogenesis. In addition to cellular proliferation, our data also implicate molecular pathways involving differentiation, Ras signaling, and protein synthesis/degradation in mammary gland cancer susceptibility. Further studies to better delineate the specific pathways contributing to the age-related differences in rat mammary cancer susceptibility is likely to provide additional insight into mechanisms of breast carcinogenesis.

Figure 5. H&E stained sections of mammary gland from 150-day-old rats at 3 hours and 6 weeks after administration of PhIP or control vehicle (water). Original magnification, $\times 200$.

Acknowledgments

We thank Ms. Melinda Blazar for assistance with mutant frequency analysis by the Big Blue assay.

References

1. Russo J, Hu YF, Silva ID, Russo IH: Cancer risk related to mammary gland structure and development. *Microsc Res Tech* 2001, 52:204–223
2. Chodosh LA, D'Cruz CM, Gardner HP, Ha SI, Marquis ST, Rajan JV, Stairs DB, Wang JY, Wang M: Mammary gland development, reproductive history, and breast cancer risk. *Cancer Res (Suppl)* 1999, 59:1765s–1772s
3. Kelsey JL, Horn-Ross PL: Breast cancer: magnitude of the problem and descriptive epidemiology. *Epidemiol Rev* 1993, 15:7–16
4. Sakorafas GH, Krespis E, Pavlakis G: Risk estimation for breast cancer development; a clinical perspective. *Surg Oncol* 2002, 10: 183–192
5. Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, van Zwieten MJ: Biology of disease: comparative study of human and rat mammary tumorigenesis. *Lab Invest* 1990, 62:244–278
6. Carmichael A, Sami AS, Dixon JM: Breast cancer risk among the survivors of atomic bomb and patients exposed to therapeutic ionising radiation. *Eur J Surg Oncol* 2003, 29:475–497
7. Hancock SL, Tucker MA, Hoppe RT: Breast cancer after treatment of Hodgkin's disease. *J Natl Cancer Inst* 1993, 85:25–31
8. Huggins C, Grand LC, Brillantes FP: Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression. *Nature* 1961, 189:204–207
9. Russo J, Russo IH: DNA labeling index and structure of the rat mammary gland as determinants of its susceptibility to carcinogenesis. *J Natl Cancer Inst* 1978, 61:1451–1459
10. Sinha DK, Pazik JE, Dao TL: Progression of rat mammary development with age and its relationship to carcinogenesis by a chemical carcinogen. *Int J Cancer* 1983, 31:321–327
11. Grubbs CJ, Peckham JC, Cato KD: Mammary carcinogenesis in rats in relation to age at time of N-nitroso-N-methylurea administration. *J Natl Cancer Inst* 1983, 70:209–212
12. Thompson HJ, Adlakha H, Singh M: Effect of carcinogen dose and

- age at administration on induction of mammary carcinogenesis by 1-methyl-1-nitrosourea. *Carcinogenesis* 1992, 13:1535–1539
13. Dao TL: Mammary cancer induction by 7,12-dimethylbenz[a]anthracene: relation to age. *Science* 1969, 165:810–811
14. Grubbs CJ, Moon RC: Effect of age and parity upon the uptake of 9,10-dimethyl-1,2-benzanthracene-9-¹⁴C by mammary parenchymal cells of the rat. *Br J Cancer* 1975, 31:189–196
15. Janss DH, Ben TL: Age-related modification of 7, 12-dimethylbenz[a]anthracene binding to rat mammary gland DNA. *J Natl Cancer Inst* 1978, 60:173–177
16. Tay LK, Russo J: In vitro metabolism of 7, 12-dimethylbenz[a]anthracene in mammary epithelial cells from rats with different susceptibilities to carcinogenesis. *Carcinogenesis* 1983, 4:733–738
17. Tay LK, Russo J: Formation and removal of 7, 12-dimethylbenz[a]anthracene-nucleic acid adducts in rat mammary epithelial cells with different susceptibility to carcinogenesis. *Carcinogenesis* 1981, 2:1327–1333
18. Tay LK, Russo J: 7, 12-Dimethylbenz[a]anthracene-induced DNA binding and repair synthesis in susceptible and nonsusceptible mammary epithelial cells in culture. *J Natl Cancer Inst* 1981, 67:155–161
19. Tay LK, Russo J: Effect of human chorionic gonadotropin on 7, 12-dimethylbenz[a]anthracene-induced DNA binding and repair synthesis by rat mammary epithelial cells. *Chem Biol Interact* 1985, 55:13–21
20. Nagasawa H, Yanai R: Frequency of mammary cell division in relation to age: its significance to the induction of mammary tumors by carcinogen in rats. *J Natl Cancer Inst* 1974, 52:609–610
21. Russo IH, Russo J: Developmental stage of the rat mammary gland as determinant of its susceptibility to 7, 12-dimethylbenz[a]anthracene. *J Natl Cancer Inst* 1978, 61:1439–1449
22. Russo J, Wilgus G, Russo IH: Susceptibility of the mammary gland to carcinogenesis. I: differentiation of the mammary gland as determinant of tumor incidence and type of lesion. *Am J Pathol* 1979, 96:721–736
23. Russo J, Russo IH: Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. *Cancer Res* 1980, 40:2677–2687
24. Sivaraman L, Stephens LC, Markaverich BM, Clark JA, Krnacik S, Conneely OM, O'Malley BW, Medina D: Hormone-induced refractoriness to mammary carcinogenesis in Wistar-Furth rats. *Carcinogenesis* 1998, 19:1573–1581
25. Medina D, Sivaraman L, Hilsenbeck SG, Conneely O, Ginger M, Rosen J, O'Malley BW: Mechanisms of hormone prevention of breast cancer. *Ann NY Acad Sci* 2001, 52:23–35
26. Snyderwine EG: Mammary gland carcinogenesis by food-derived heterocyclic amines: metabolism and additional factors influencing carcinogenesis by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Environ Mol Mutagen* 2002, 39:165–170
27. Sinha R, Gustafson DR, Kulldorff M, Wen W-Q, Cerhan JR, Zheng W: 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, a carcinogen in high-temperature-cooked meat, and breast cancer risk. *J Natl Cancer Inst* 2000, 92:1352–1354
28. Ghoshal A, Preisegger K-H, Takayama S, Thorgeirsson SS, Snyderwine EG: Induction of mammary tumors in female Sprague-Dawley rats by the food-derived carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, and effect of dietary fat. *Carcinogenesis* 1994, 15:2429–2433
29. Snyderwine EG, Thorgeirsson UP, Venugopal M, Roberts-Thomson SJ: Mammary gland carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in Sprague-Dawley rats on high- and low-fat diets. *Nutr Cancer* 1998, 31:160–167
30. Yu M, Jones ML, Gong M, Sinha R, Schut HAJ, Snyderwine EG: Mutagenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in the mammary gland of Big Blue rats on high- and low-fat diets. *Carcinogenesis* 2002, 23:877–884
31. Schut HAJ, Herzog CH: Formation of DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in male Fischer 344 rats. *Cancer Lett* 1992, 67:117–124
32. Ghoshal A, Davis CD, Schut HAJ, Snyderwine EG: Possible mechanisms for PhIP-DNA adduct formation in the mammary gland of female Sprague-Dawley rats. *Carcinogenesis* 1995, 16:2725–2731
33. Edgerton ME, Taylor R, Powell JL, Hunter L, Simon R, Liu ET: A bioinformatics tool to select sequences for microarray studies of mouse models of oncogenesis. *Bioinformatics* 2002, 18:774–775
34. Desai KV, Kavanaugh CJ, Calvo A, Green JE: Chipping away at breast cancer: insights from microarray studies of human and mouse mammary cancer. *Endocr Relat Cancer* 2002, 9:207–220
35. Rosen JM, Woo SL, Comstock JP: Regulation of casein messenger RNA during the development of the rat mammary gland. *Biochemistry* 1975, 14:2895–2903
36. Oshima K, Aoki N, Kato T, Kitajima K, Matsuda T: Secretion of a peripheral membrane protein, MFG-E8, as a complex with membrane vesicles. *Eur J Biochem* 2002, 269:1209–1218
37. Charlwood J, Hanrahan S, Tyldesley R, Langridge J, Dwek M, Camilleri P: Use of proteomic methodology for the characterization of human milk fat globular membrane proteins. *Anal Biochem* 2002, 301:314–324
38. Qu X, Zhai Y, Wei H, Zhang C, Xing G, Yu Y, He F: Characterization and expression of three novel differentiation-related genes belong to the human NDRG gene family. *Mol Cell Biochem* 2002, 229:35–44
39. Rosen JM, Zahnow C, Kazansky A, Raught B: Composite response elements mediate hormonal and developmental regulation of milk protein gene expression. *Biochem Soc Symp* 1998, 63:101–113
40. Cavaletto M, Giuffrida MG, Fortunato D, Gardano L, Dellavalle G, Napolitano L, Giunta C, Bertino E, Fabris C, Conti A: A proteomic approach to evaluate the butyrophilin gene family expression in human milk fat globule membrane. *Proteomics* 2002, 2:850–856
41. Oxford G, Theodorescu D: Ras superfamily monomeric G proteins in carcinoma cell motility. *Cancer Lett* 2003, 189:117–128
42. Yu M, Snyderwine EG: H-ras oncogene mutations during development of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced rat mammary gland cancer. *Carcinogenesis* 2002, 23:2123–2128
43. Seth P, Porter D, Lahti-Domenici J, Geng Y, Richardson A, Polyak K: Cellular and molecular targets of estrogen in normal human breast tissue. *Cancer Res* 2002, 62:4540–4544
44. Voigt S, Jungnickel B, Hartmann E, Rapoport TA: Signal sequence-dependent function of the TRAM protein during early phases of protein transport across the endoplasmic reticulum membrane. *J Cell Biol* 1996, 134:25–35
45. Greenfield JJ, High S: The Sec61 complex is located in both the ER and the ER-Golgi intermediate compartment. *J Cell Sci* 1999, 112:1477–1486
46. Snyderwine EG, Davis CD, Schut HAJ, Roberts-Thomson SJ: Proliferation, development and DNA adduct levels in the mammary gland of rats given 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and a high-fat diet. *Carcinogenesis* 1998, 19:1209–1215
47. Snyderwine EG, Venugopal M, Yu M: Mammary gland carcinogenesis by food-derived heterocyclic amines and studies on the mechanisms of carcinogenesis of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Mutat Res* 2002, 506–507:145–152
48. Schut HAJ, Snyderwine EG: DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. *Carcinogenesis* 1999, 20:353–368
49. Bielas JH, Heddle JA: Proliferation is necessary for both repair and mutation in transgenic mouse cells. *Proc Natl Acad Sci USA* 2000, 97:11391–11396
50. Masso-Welch PA, Darcy KM, Stangle-Castor NC, Ip MM: A developmental atlas of rat mammary gland histology. *J Mammary Gland Biol Neoplasia* 2000, 5:165–185
51. Russo J, Saby J, Isenberg WM, Russo IH: Pathogenesis of mammary carcinomas induced in rats by 7, 12-dimethylbenz[a]anthracene. *J Natl Cancer Inst* 1977, 59:435–445
52. Ginger MR, Gonzalez-Rimbau MF, Gay JP, Rosen JM: Persistent changes in gene expression induced by estrogen and progesterone in the rat mammary gland. *Mol Endocrinol* 2001, 15:1993–2009
53. Ichinose RR, Nandi S: Influence of hormones on lobulo-alveolar differentiation of mouse mammary gland in vitro. *J Endocrinol* 1966, 35:331–340
54. Shan L, Rouhani SA, Schut HAJ, Snyderwine EG: 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) modulates lactogenic hormone-mediated differentiation and gene expression in HC11 mouse mammary epithelial cells. *Cell Growth Differ* 2001, 12:649–656
55. Chen FW, Ioannou YA: Ribosomal proteins in cell proliferation and apoptosis. *Int Rev Immunol* 1999, 18:429–448
56. Ruggero D, Pandolfi P: Does the ribosome translate cancer? *Nat Rev Cancer* 2003, 3:179–192
57. Frigerio JM, Dagorn JC, Iovanna JL: Cloning, sequencing, and ex-

- pression of the L5, L21, L27a, L28, S5, S9, S10, and S29 human ribosomal protein mRNAs. *Biochim Biophys Acta* 1995, 1262:64–68
58. Pogue-Geile K, Geiser JR, Shu M, Miller C, Wool IG, Meisler AI, Pipas JM: Ribosomal protein genes are overexpressed in colorectal cancer: isolation of a cDNA clone encoding the human S3 ribosomal protein. *Mol Cell Biol* 1991, 11:3842–3849
59. Hamel FG, Upward JL, Siford GL, Duckworth WC: Inhibition of proteasome activity by selected amino acids. *Metabolism* 2003, 52:810–814
60. Wojcik C: Regulation of apoptosis by the ubiquitin and proteasome pathway. *J Cell Mol Med* 2002, 6:25–48
61. Delcros JG, Floc'h MB, Prigent C, Arlot-Bonnemains Y: Proteasome inhibitors as therapeutic agents: current and future strategies. *Curr Med Chem* 2003, 10:479–503
62. Grantham WD, Zhu XO, Wei Lin J, van Oosterum M, Taylor R, Edwards M: The role of heat shock proteins in mammalian differentiation and development. *Environ Med* 1999, 43:79–87
63. Parcellier A, Gurbuxani S, Schmitt E, Solary E, Garrido C: Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. *Biochem Biophys Res Commun* 2003, 304:505–512
64. Conroy SE, Latchman DS: Do heat shock proteins have a role in breast cancer? *Br J Cancer* 1996, 74:717–721
65. Malaney S, Daly RJ: The Ras signaling pathway in mammary tumorigenesis and metastasis. *J Mammary Gland Biol Neoplasia* 2001, 6:101–113
66. Osborne MP, Ruperto JF, Crowe JP, Rosen PP, Telang NT: Effect of tamoxifen on preneoplastic cell proliferation in N-nitroso-N-methylurea-induced mammary carcinogenesis. *Cancer Res* 1992, 52:1477–1480
67. Thompson HJ, McGinley JN, Wolfe P, Singh M, Steele VE, Kelloff GJ: Temporal sequence of mammary intraductal proliferations, ductal carcinomas in situ, and adenocarcinomas induced by 1-methyl-1-nitrosourea in rats. *Carcinogenesis* 1998, 19:2181–2185